SULFATION IS RATE LIMITING IN THE FUTILE CYCLING BETWEEN ESTRONE AND ESTRONE SULFATE IN ENRICHED PERIPORTAL AND PERIVENOUS RAT HEPATOCYTES

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ABSTRACT:

The metabolic activities and tissue binding of estrone (E1) and estrone sulfate (E1S) on futile cycling were examined. Desulfation of E1S in the 9000g supernatant fraction (S9) of peripoortal (PP) and perivenous (PV) rat hepatocytes were of similar \( V_{\max}^{E1S} \) (2.9 ± 1.0 and 2.4 ± 0.9 nmol/min/mg of S9 protein), \( K_{m}^{E1S} \) (30.4 ± 8.3 and 34.8 ± 6.6 \( \mu \)M), and desulfation intrinsic clearances (\( V_{\max}^{E1S}/K_{m}^{E1S} \) of 77 and 55 \( \mu \)l/min/10^6 cells). The intrinsic clearance towards E1 sulfation (1 \( \mu \)M) in cytosolar preparations of PP hepatocytes was 4 times that of PP hepatocytes (\( V_{\max}^{E1}/K_{m}^{E1} \) of 26.4 ± 9.5 versus 6.1 ± 2.2 \( \mu \)l/min/mg of cytosolic protein or 13 ± 5 versus 3.1 ± 1.1 \( \mu \)l/min/10^6 cells). The observation was consistent with the immunolocalization of estrogen sulfotransferase (PV/PP ratio of 3.4 ± 1.1) but not hydroxysteroid sulfotransferase (PV/PP ratio of 0.29 ± 0.21) nor phenol sulfo transferase (PV/PP ratio of 1.13 ± 0.23). Upon incubation of E1S (1–125 \( \mu \)M) with hepatocytes (30 min), higher concentrations of E1S and E1 were observed within PP than in PV cells, and saturation was evident at the higher concentrations. Based on the in vitro metabolic and tissue binding parameters for E1S and E1 and the published zonal uptake clearances of E1S (116 \( \mu \)l/min/10^6 cells) by PP and PV cells was rapid and similar, and E1 sulfation was the slowest step in futile cycling. The greater metabolism of E1 in PV region led to higher levels of E1 and E1S in PP hepatocytes, and the nonlinear uptake, binding, and vesicular accumulation of E1S resulted in different \( t_{1/2} \) values for E1S and E1.

Estrone sulfate (E1S) is one of the compounds used in hormone replacement therapy. The activity of E1S results from the release of active estrone (E1) through desulfation in liver, and E1 can be sulfated back to the inactive E1S. Typically, the rate of sulfation of E1 is measured, but the observation may be erroneously based on the net rate of formation of E1S. Since sulfocojunction plays an important role in the deactivation and elimination of E1, it is critical to consider the roles of sulfation and desulfation on the duration of activity of E1S.

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Abbreviations used are: E1S, estrone sulfate; E1, estrone; E2, estradiol; E1G, estrone glucuronide; PP, peripoortal; PV, perivenous; PAGE, polacycmylamide gel electrophoresis; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; HPLC, high-performance liquid chromatography; S9, 9000g supernatant fraction; rSULT1A1, estrogen sulfotransferase.

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 Estrone sulfate, a membrane-bound enzyme with a pH optimum of 7.4 is mainly responsible for the desulfation of E1S and exhibits its highest activity in the liver (Milewich et al., 1984). Estrone sulfatase is also known as arylsulfatase C, a microsomal enzyme that copurifies with steroid sulfatase and cleaves the sulfate moiety of several 3-hydroxysteroid sulfates (Dolly et al., 1972). In female rats, an abundance of estrone sulfatase activity was found in both the endoplasmic reticulum and nucleus (Zhu et al., 1998). Previous studies suggest that desulfation of 4-methylumbelliferyl sulfate by arylsulfatase C was homogeneous across the rat liver (Anundi et al., 1986) and human liver tissues (El Mouelhi and Kauffman, 1986).

The phenomenon of futile cycling between E1 and E1S has yet to be

estrogens. When sulfation or desulfation is altered in disease states, the apparent formation of E1S will be further modulated.

One of the key enzymes responsible for the sulfation of E1 is estrogen sulfotransferase, a cytosolic enzyme that also sulfates estradiol (E2) in the presence of the obligate cosubstrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Both human hydroxysteroid and phenol sulfotransferases are known to sulfate estrogens (Falany et al., 1994), but these possess lower affinities towards E1 (Falany et al., 1994, 1995). Thus, under physiological conditions, E1 is more likely to be sulfated predominantly by estrogen sulfotransferase than by other isomers of sulfotransferases. Previous studies had revealed that estrogen sulfotransferase was localized more abundantly in the PV than in the PP region of the rat liver. On the other hand, hydroxysteroid sulfotransferase was predominantly present in the PP hepatocytes, and the phenol sulfotransferase was evenly distributed in the liver acinus (Tosh et al., 1996).
viewed in conjunction with hepatic transport of E₁ and E₁S and other metabolic pathways of E₁. Transport of E₁S in rat liver is found to be mediated by the organic anion-transporting polypeptides, Oatp1 (Jacquemin et al., 1994), Oatp2 (Noé et al., 1997), and Oatp4 (Cattori et al., 2000); the multispecific organic anion transporter 3, OAT3 (Kusu-hara et al., 1999); and the sodium-dependent taurocholate-cotransporting polypeptide. Ntcp (Hagenbuch et al., 1991). The $K_m$ values for the uptake of E₁S mediated by Oatp1 (Bossuyt et al., 1996), Oatp2 (Noé et al., 1997), and Ntcp (Schroeder et al., 1998) expressed in the Xenopus laevis oocytes were around 4.5 to 27 μM, showing that transport is of high affinity. Transport of E₁S was defined by these sinusoidal transporters and passive diffusion and was found to be similar among perivenous (PV) and perportal (PP) rat hepatocytes (Tan et al., 1999).

The objective of this study was to highlight the importance of transport, metabolism, and zonal aspects to understand their interplay on the futile cycling of estrogens in intact hepatocytes and, ultimately, the whole organ. In this article, metabolic activities in subcellular fractions of enriched PP and PV rat hepatocytes were assessed and in turn related to cellular expressions of rat liver estrone sulfatase and estrone sulfortransferase. These in vitro metabolic parameters and previously obtained transport parameters on hepatocyte uptake (Tan et al., 1999) were then used to describe the futile cycling between E₁S and E₁ in intact cells when E₁S (1, 5, 25, and 125 μM) was incubated with PP and PV hepatocytes.

**Experimental Procedures**

**Materials.** [6,7,3H]E₁S (ammonium salt; specific activity, 53 Ci/mmole), [6,7,3H]E₁ (specific activity, 40.6 Ci/mmole), and [4-14C]E₁ (specific activity, 56.6 Ci/mmole) were purchased from NEN Life Science Products (Boston, MA). The radiochemical purities, as found by high-performance liquid chromatography (HPLC) or thin-layer chromatography, were greater than 95%. Unlabelled E₁S, E₁, PAPS, and bovine serum albumin (fraction V) were purchased.

56.6 Ci/mol) were purchased from NEN Life Science Products (Boston, MA).

The estrone sulfotransferase activities in the S9 of zonal hepatocytes, which were preincubated at 37°C for 5 min, were added to mixtures of PAPS, E₁, and [3H]E₁ (2.0 ± 0.1 × 10⁶ dpm/ml) to result in 1 μM E₁ and 100 μM PAPS in 1 ml of Tris-HCl buffer (25 mM) at pH 7.4. To ensure sufficient cosubstrate for the reaction, the PAPS concentration chosen was higher than that reported for the rat liver (70 nmol/g of liver or 117 μM PAPS in cell water) (Brzezniacka et al., 1987). Samples (0.2 ml) were removed at 6 min, a predetermined time in which E₁ sulfation was linear with time. The samples were added to 0.8 ml of acetonitrile containing 4 μM danazol.

**Estrone Sulfatase Activity.** Estrone sulfatase activity in the S9 of zonal hepatocytes, which were preincubated at 37°C for 5 min, were added to mixtures of PAPS, E₁, and [3H]E₁ (2.0 ± 0.1 × 10⁶ dpm/ml) to result in 1 μM E₁ and 100 μM PAPS in 1 ml of Tris-HCl buffer (25 mM) at pH 7.4. To ensure sufficient cosubstrate for the reaction, the PAPS concentration chosen was higher than that reported for the rat liver (70 nmol/g of liver or 117 μM PAPS in cell water) (Brzezniacka et al., 1987). Samples (0.2 ml) were removed at 6 min, a predetermined time in which E₁ sulfation was linear with time. The samples were added to 0.8 ml of acetonitrile containing 4 μM danazol.

**Metabolism of E₁S in Intact Zonal Hepatocytes.** Zonal hepatocyte suspensions (2 × 10⁶ cells/ml, preincubated for 10 min at 37°C in the incubation buffer, were added to equimolar concentrations of unlabelled E₁S and [3H]E₁S (5.1 ± 0.3 × 10⁶ dpm/ml) prepared in incubation buffer to result in 1, 5, 25, and 125 μM E₁S in 10⁶ cells/ml. Two samples were retrieved at various times (1–30 min) from the incubation mixture. The first sample (100 μl) was immediately extracted with 0.4 ml of acetonitrile containing 4 μM danazol, and the second sample (150 μl) was placed immediately into a polyethylene microcentrifuge tube (300 μl) containing 100 μl of 1-bromomodecane. Upon centrifugation at 9000g (2 s), hepatocytes were removed into the layer of 1-bromomodecane, and 100 μl of the resultant extracellular medium remaining on top was removed into a 1.5-ml microcentrifuge tube containing 4 μM danazol in 0.4 ml of acetonitrile. Subsequently, the content of E₁S and E₁ in the incubation mixture and extracellular medium were assayed by HPLC. The amount of E₁S or E₁ in the cellular space was estimated by the difference of the quantities in the incubation mixture and extracellular medium. The difference in mass between the administered amount and the sum of E₁S and E₁ provided an estimate of the formation of other E₁ metabolites (M; estrone glucuronide or E₁G, estradiol or E₂, and its conjugates) at various times.

**Protein Binding/Metabolism in Extracellular Medium.** During the preparation of isolated hepatocytes, protein debris from suspending dead cells (0.16 ± 0.06 mg/10⁶ cells) was found to persist routinely in the incubation mixture despite the washings; centrifugation with percoll failed to remove the presence of the protein debris fragments. In view of the tight binding of E₁S and E₁ to albumin (Rosemhal et al., 1972; Rao, 1998), binding of E₁S and E₁ to the protein debris in the extracellular medium was estimated by ultrafiltration (Centricon 3, Amicon Inc., MA). Solutions containing [3H]E₁ (8.5 × 10⁶ dpm/ml) or [3H]E₁S (4.9 ± 2.4 × 10⁶ dpm/ml) plus E₁S (0.8–2.5 μM) were added to blank extracellular medium of the incubation mixture, which was prepared in the absence of drug. After incubation of the mixture for 10 min at 37°C, an aliquot (2 ml) was removed into a Centricon tube and centrifuged at 2500g for 20 min at 37°C. Liquid scintillation fluor (5 ml, Ready Safe, Beckman Coulter, Mississauga, ON, Canada) was added to the original mixture (0.2 ml) and the resulting filtrate (0.2 ml) in different mixing vials, then quantified by liquid scintillation spectrometry (model LS6800, Beckman Coulter). Leakage of protein through the Centricon filter was less than 0.5% of the mixture solution. Desulfation of E₁S and metabolism of E₁ within the extracellular medium were less than 1% over the experimental time and were deemed insignificant.

**Immunoblot Analysis.** Lysates, centrifuged at 100,000g for 60 min at 4°C, and the cytosolic fraction of zonal hepatocytes, prepared as described by Tirona et al. (1999), was used for immunoblot analysis. Aliquots containing 10 μg of protein were resolved by SDS-PAGE in a 12% polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. Primary rabbit an-
tibodies (anti-rSULT1A1 at 1:20,000, anti-rSULT2A1 at 1:10,000, and anti-rSULT1E1 at 1:20,000 dilution) were then incubated with the blots for 1 h at room temperature. Finally, goat anti-rabbit IgG horseradish peroxidase conjugate (1:40,000) was used as the secondary antibody, and the immunocomplexes were detected by chemiluminescence (Amersham). The intensity of the protein band was integrated using the NIH Image software (http://rsb.info.nih.gov).

Protein Assay. In all preparations, protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

HPLC Analysis. Liquid chromatography was performed on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a SCL-10A system controller, a SPD-10A-XI automatic injector, and a CR-4A chromaticap integrator. Separation was carried out by a 10-μm Bondapak C18 reversed-phase column (39 cm × 300-mm i.d.: Waters, Milford, MA). A binary gradient consisting of mobile phase A (10 mM ammonium acetate, pH 7.5) and mobile phase B (acetonitrile) was used at a constant flow rate of 1 ml/min for the separation of E1G, E1S, E1, E2, and danazol. Initially, the wavelength of detection was set at 270 nm for the detection of E1G and E1S, and the mobile phase was linearly increased from 10% B to 50% B in 15 min. Then the mobile phase was maintained at 50% B for 10 min, and the wavelength was altered to 285 nm at 20 min for the detection of E1G and E2. At 25 min, the mobile phase was linearly increased to 75% B in 2 min and was maintained for another 10 min before being brought back down to 10% B over the next 5 min. The mobile phase was maintained at 10% B for an additional 5 min to re-equilibrate the column. The retention times were as follows: E1G, 14 min; E1S, 16 min; E1, 24 min; E2, 26 min; and danazol, 34 min.

Good correspondence was observed between the eluted radioactivity ([3]H]E1S and [3]H]E1), which was collected at 0.5-min intervals after sample injection and UV absorbance. No carryover of radioactivity was observed for the compounds of interest despite a delay of 0.5 min between UV detection and radioassay. Therefore, the delay time was incorporated to the automated fraction collector (model 202, Gilson Medical Electronics, Middleton, WI). After the addition of 10 ml of Ready Safe (Beckman Coulter), the collected fractions were quantified by liquid scintillation spectrometry (model LS6800, Beckman Coulter).

Kinetic Analysis for Extracellular Binding of E1S and E1. The binding of E1S and E1 to proteins that were present in the extracellular medium was described by the Langmuir binding isotherm (eq. 1), where the bound concentration of extracellular E1S ([E1Sbound]ec) was related to the unbound concentration ([E1Sunbound]ec) by the binding dissociation constant, Kd[S], and the product of number of binding sites (n[S]ec) and the total protein concentration in the extracellular medium ([Ptotal]ec). The kinetic constants were obtained by regression of [E1Sbound]ec versus [E1Sunbound]ec according to eq. 1 with use of the fitting software package SCIENTIST (version 2; MicroMath Scientific Software, Salt Lake City, UT) and an optimized weighting scheme (1/observation3):

\[ [E1S_{\text{bound,ec}}] = \frac{n[S]_{\text{ec}}[E1S_{\text{unbound,ec}}]}{K_d[S] + [E1S_{\text{unbound,ec}}]} . \] (1)

After obtaining Kd[S] and n[S]ec from eq. 1, eqs. 2 and 3 were used to estimate the tissue binding of E1S in the hepatocyte from known total protein concentration in cell-homogenate [Ptotal].

\[ n[E1S_{\text{total}}] = \frac{n[S]_{\text{ec}}[P_{\text{total}}]}{[P_{\text{total,ec}}]} , \] (2)

\[ [E1S_{\text{bound,tl}}] = \frac{n[S]_{\text{ec}}[E1S_{\text{unbound,ec}}]}{K_d[S] + [E1S_{\text{unbound,ec}}]} , \] (3)

\[ [E1S_{\text{unbound,tl}}] = [E1S_{\text{total}}] - [E1S_{\text{bound,tl}}] . \] (4)

Kinetic Modeling of E1S and E1 Disposition in Intact Zonal Hepatocytes. A cellular, kinetic model that considered protein binding in both extracellular and cellular spaces, transport, metabolism, interconversion, and an intracellular vesicular compartment (Fig. 1) best described the concentration- and time-dependent data of E1S and E1 in enriched PP and PV hepatocytes. Inclusion of binding in both cellular and extracellular medium is justified in view of the demonstrable binding but lack of metabolism in extracellular medium, which contained protein debris from cells. E1S binding and debinding to protein are denoted by on- and off-rate constants, koff[S] and kon[S], respectively; these constants and the ratio Kd[S] (koff[S]/kon[S]) are expected to be identical for both extracellular and cell media. However, the protein concentration or [Ptotal]ec in the extracellular medium is only a fraction of that in cells since the [Ptotal]c is much higher. The effective binding concentration in cell was determined by multiplication of the ratio of protein concentrations in cell/debris ([Ptotal]/[Ptotal,ec]) with n[S]ec[Ptotal,ec] according to eq. 2. For the sake of simplicity, the unbound concentration of E1S is represented by the product of the unbound fractions of E1 (fS and fS/ec represent the unbound fraction of E1 in the cell and the extracellular medium, respectively) and the total concentration of E1. Since the canalicular membrane on the cell surface that mediates excretion of anions is known to internalize to form vesicles shortly after hepatocyte isolation (Oude Elferink et al., 1993), a similar phenomenon was postulated to exist for E1S accumulation into vesicles. A vesicular compartment is included for E1S since E1S undergoes demonstrable biliary excretion in the perfused rat liver (Tan et al., 2001).

Previously obtained parameters on transport were scaled up and used in the fitting procedure. The transport parameters for E1S (Vmax and Pdiff[S]) obtained from our previous hepatocyte uptake study (Tan et al., 1999) were...
scaled up with the factor $\phi = 1.6$ mg of protein/10^6 cells; Mahler and Cordes, 1966; Lin et al., 1980). Based on these transport parameters of E_1 S, the uptake of E_1 S was mainly attributed to carrier-mediated transport, although the minor bidirectional flux of E_1 S (P_{net}^{E_1 S}) also contributed to E_1 S transport. Because uptake of E_1 S was too rapid for proper characterization of transport constants in hepatocyte uptake studies, the observed transport clearance for E_1 S, $P_{net}^{E_1 S}$, is assumed to exist for the permeation of E_1 S at all concentrations. The in vitro parameter for the desulfation of E_1 S ($V_{max}^{E_1 S}$) and the linear sulfation intrinsic clearance of E_1 S ($K_{m}^{E_1 S}$) were scaled up with factors $\alpha$ and $\beta$ ($\alpha = 0.8$ mg of S9 protein/10^6 cells; $\beta = 0.5$ mg of cytosolic protein/10^6 cells; Mahler and Cordes, 1966; Lin et al., 1980), respectively. It must be noted that the metabolic intrinsic clearance for estrone sulfation, $C_{str}^{E_1 S}$, could only be defined within a narrow concentration range of E_1 S in vitro because of poor aqueous solubility, and this was used to relate $C_{str}^{E_1 S}$ to the ratio $V_{max}^{E_1 S}/C_{str}^{E_1 S}$. Since E_1 S is known to form other metabolites (M), denoting the mixture of E_1 G, estradiol, estriol, and other estrogen conjugates; Holler et al., 1976), a simplified metabolic scheme (Fig. 1) was used to describe the formation of all M with the pooled metabolic constants, $V_{max}^{M}$ and $K_{m}^{M}$. The intrinsic clearance of E_1 S entry into vesicles is denoted by $C_{str}^{E_1 S}$. The PP and PV cell volumes ($V_{c}$) were assumed to be 2.5 and 2.4 $\mu$m^3 cells, respectively (Garcia-Ruiz et al., 1994), and the extracellular volume ($V_{e}$) was obtained by the difference [total volume of the preparation (1 ml) − $V_{c}$].

**Fitting.** Mass balanced rate equations (see Appendix) were written to describe events of the cellular kinetic model (Fig. 1). Fitting was performed by the software package SCIENTIST based on experimentally obtained binding, metabolic, and transport parameters (Table 1). The parameters—transport clearance of E_1 S ($P_{net}^{E_1 S}$), the Michaelis-Menten constant for uptake of E_1 S into hepatocytes ($K_{m}^{E_1 S}$), the vesicular intrinsic clearance for accumulation of E_1 S ($K_{m}^{E_1 S}$), the pooled kinetic constants for formation of the composite of E_1 metabolites M ($V_{max}^{M}$ and $K_{m}^{M}$), the kinetic constants for E_1 sulfation ($V_{max}^{E_1 S}$ and $K_{m}^{E_1 S}$), the latter constrained as $V_{max}^{E_1 S} = E_1 S/\mu$mol/min/E_1 S, and the tissue binding of E_1 S—were optimized by least-square fitting with appropriate weighting schemes of 1/observation (for data increasing in value) and 1/observation^2 (for data decreasing in value). The goodness of fit was viewed with respect to the coefficient of variation (standard deviation of parameter estimate/parameter value), the residual plot, and the Model Selection Criterion (MSC).

**Statistics.** All data were presented as the mean ± S.D., and the means were compared by use of ANOVA, with the $P$ value of 0.05 as the level of significance. A paired $t$ test was used to compare the means for the data on zonal lysates since the same liver was used for the preparation of both PP and PV lysates; the level of significance was set at 0.05.

**Results**

**Biochemical Characterization of Zonal Hepatocytes and Lysates.** The marker enzymes, alanine aminotransferase and glutamine synthetase, verified that enriched PP and PV hepatocytes and lysates were isolated from different acinar regions of the rat liver. The PP/PV ratios of the marker enzyme alanine aminotransferase for zonal hepatocytes and zonal lysates were 3.3 ± 0.2 and 7.4 ± 2.5, respectively. The acinar gradient of alanine aminotransferase content in zonal lysates was steeper because the preparations were obtained from the most distal and proximal acinar regions of the rat liver. However, the PP/PV ratios of the marker enzyme glutamine synthetase for the zonal hepatocytes and lysates were 0.027 ± 0.023 and 0.029 ± 0.013, respectively, and were similar. These PP/PV ratios were in agreement with those reported by others (Lindros and Penttilä, 1985; Quistorff and Grunnet, 1987; Tosh et al., 1996; Tirona et al., 1999).

**Desulfation of E_1 S in the Zonal S9 Preparations.** Preliminary study failed to show E_1 sulfation in absence of PAPS within the zonal S9 preparations. Therefore, the rate of E_1 formation from E_1 S in S9 represented the true desulfation rate. The results were best fit to the Michaelis-Menten equation (Table 1 and Fig. 2), yielding similar $K_{m}^{E_1 S}$ (30.3 to 34.6 $\mu$mol/min/S9 protein) and $V_{max}^{E_1 S}$ (2.4 to 2.9 nmol/min/S9 protein) values for estrone desulfation in the PP and the PV preparations (ANOVA, $P > 0.05$). The $K_{m}^{E_1 S}$ was much lower in relation to that reported for another well studied sulfate conjugate, 4-methylumbelliferone (Chiba and Pang, 1993).

**E_1 Sulfation in Zonal Lysates and the Cytosol of Zonal Hepatocytes.** Estrone sulfotransferase activity was detected in zonal lysates and cytosolic fractions of PP and PV hepatocytes in the presence of PAPS. Desulfation of E_1 S in zonal lysate and zonal cytosol was less than 1% over the experimental time and was negligible. Hence in both lysate and cytosol, the observations reflected the true and not the net sulfation activity of E_1 sulfotransferases. The activity was significantly higher in the PV region than the PP region (Table 2). The PV/PP ratios of estrone sulfation activities in the cytosolic fractions and lysates were 4.3 ± 2.2 and 3.1 ± 1.8, respectively.

**Immunoblot of rSULT1A1, rSULT2A1, and rSULT1E1 in Zonal Hepatocytes and Zonal Lysates.** The rSULT1E1 protein in the cytosolic fraction of PV hepatocytes was 3.4 ± 1.1 times that of PP and paralleled the results obtained previously for CYP1A2, a PV marker within the same cell preparations (PV/PP ratio of 4.1 ± 3.3; Tirona et al., 1999). On the other hand, the rSULT2A1 protein in PP hepatocytes was 3.5 ± 2.7 times that of the PV hepatocytes, and the rSULT1A1 protein was not significantly different (ANOVA, $P > 0.05$; PV/PP ratio of 1.13 ± 0.23) among zonal hepatocytes (Fig. 3). Trends for the zonal lysates remained similar to those of the zonal hepatocytes: the rSULT1A1 protein in the PV lysates was 4.0 ± 3.0 times that of the PP lysates. The rSULT2A1 protein was exclusively found in the PP lysates, and the rSULT1A1 protein was again present evenly among zonal lysates (Fig. 4).

**Protein Binding of E_1 S and E_1.** The binding data of E_1 S (0.8–250 $\mu$mol in extracellular medium were best described by the Langmuir binding isotherm (eq. 1). Assuming that the molecular mass of binding proteins were around 82 kDa (the molecular mass of estrogen binding protein; Rao, 1998), the $n^E_1 S/P_{total}^{E_1 S}$ and the $K_{D}^{E_1 S}$ for E_1 S

<table>
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<th>TABLE 1</th>
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<tr>
<td><strong>Desulfation kinetic parameters of E_1 S in zonal S9 preparation at 37°C (n = 4)</strong></td>
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<tr>
<td>PP</td>
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<td>PV</td>
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**Fig. 2.** Rates of desulfation (mean ± S.D.) for E_1 S in the S9 fractions of the PP and PV hepatocytes at 37°C (n = 4).

Lines, fitted lines based on the Michaelis-Menten equation with an optimal weighting scheme of unity.
were estimated to be 23.5 ± 3.0 and 23.4 ± 4.5 μM, respectively, and the number of binding sites (K_D) was 12 ± 1.5, based on the measured extracellular protein concentration of 0.16 ± 0.03 mg/ml (Fig. 5A). The unbound fractions of E_1 S at 1 and 125 μM in the extracellular medium were around 0.5 and 0.8, respectively. In tissue, where the cellular protein concentration (1.6 ± 0.3 mg/ml) was about 10 times the extracellular concentration, the unbound fractions of E_1 S in tissues were calculated according to eq. 3, based on the K_D and K_M. The unbound fractions of E_1 S in tissue were predicted to vary from 0.1 to 0.7, as shown in Fig. 5B.

When the binding study was repeated for tracer E_1 S, a much higher unbound fraction of 0.84 ± 0.03 was found. Again, due to the poor aqueous solubility of E_1 S, binding at higher E_1 concentrations could not be studied.

**TABLE 2**

Sulfation Intrinsic Clearance of Estrone and zonal lysates (n = 4) in the presence of the cosubstrate PAPS (700 μM)

<table>
<thead>
<tr>
<th>Zonal Hepatocytes</th>
<th>PP/PP Ratio</th>
<th>μm/min/mg protein</th>
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<tr>
<td>Zonal Lysates</td>
<td></td>
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<tr>
<td>Periportal (LP)</td>
<td>8.3 ± 4.3</td>
<td>0.32</td>
</tr>
<tr>
<td>Perivenous (LV)</td>
<td>25.9 ± 6.8**</td>
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* Statistically significant from periportal (ANOVA, P < 0.05).
** Statistically significant from periportal (paired t test, P < 0.05).

**Metabolism of E_1 S in Intact Zonal Hepatocytes.** Concentration-dependent metabolism of E_1 S was observed in intact PP and PV hepatocytes (Fig. 6A). For both PP and PV hepatocyte preparations, biphasic elimination patterns were observed for the lower concentrations of E_1 S (<25 μM), whereas monoeponential decay profiles were observed at the highest concentration of E_1 S used (125 μM). The patterns of E_1 S in extracellular medium paralleled those in the incubation mixture (Fig. 6B) and were similar for both PP and PV cells. However, the pattern differed dramatically in the cell wherein cellular concentrations of E_1 S were much higher than those extracellularly (Fig. 6C), yielding apparent tissue to medium partitioning ratios of greater than unity in both PP and PV cells (Fig. 7A). The apparent partition coefficients of E_1 S at equilibrium decreased with increasing E_1 S concentration and were similar for both PP and PV cells (Fig. 7B). As shown in Table 3, nonlinear kinetics were shown to exist with increasing E_1 S doses. Values of the AUC of E_1 S were higher in PP than in PV hepatocytes, although statistical significance was not found (Table 3). The apparent clearance of E_1 S [dose/AUC ec] decreased with increasing dose and was lower in PP hepatocytes than in PV hepatocytes. Again, statistical difference was not found due to the large interanimal variability.

Patterns of E_1 in the extracellular medium (Fig. 6E) and incubation mixture (Fig. 6D) were similar to those for the cell (Fig. 6F), but the decay of E_1 was faster than that of E_1 S (Fig. 6). The terminal half-lives of E_1 differed for each dose (compare Fig. 6C with 6F). At the lower initial concentrations of E_1 S used for incubation with hepatocytes, greater E_1 levels existed in PP hepatocytes than in PV hepatocytes. Values of the AUC of E_1 were higher in PP than in PV
hepatocytes, and statistical significance was found for AUC$^{E_1S}_{EC}(0 \rightarrow 30$ min) for the lower concentrations of E$1_S$ used for this study (Table 3). At higher initial concentrations of E$1_S$ (>5 μM), the decay half-life of E$1$ in the cell was more prolonged, suggesting that the enzymes for the metabolism of estrone had become saturated.

**Fitted Results for E$1_S$ and E$1$ in Intact Zonal Hepatocytes with the Kinetic Model.** When simultaneous fitting was performed on the total, extracellular, and cellular E$1_S$ and E$1$ data for each set of experiments consisting of four E$1_S$ initial concentrations and the same pool of hepatocytes, good fits were obtained, although high coefficients of variation were found associated with the fitted parameters. The optimized fit that considered tissue binding and vesicular storage of E$1_S$ is presented in Fig. 6, and the mean of the optimized parameters of five experiments and the assigned parameters are summarized in Table 4.

The uptake constant of E$1_S$, $K_{in}^{E_1S}$, when optimized (10–12 μM), was only half of the in vitro value (24 μM), showing that the hepatocyte uptake clearance (CL$^{E_1S}_{uptake}$; 246–282 μl/min/10$^6$ cells or 31–35 ml/min/g of liver; Table 5), although of high value under first-order conditions, was readily saturated. The hepatocyte uptake clearance for E$1$ that is assumed to mediate bidirectional transport was even faster (1463–1484 μl/min/10$^6$ cells). These values suggest that under first-order conditions, transport of the estrogenic compounds are flow-rate limited in the rat liver.

The $V_{max}^{E_1S}$ for estrone sulfation was low, differing in both PP and PV cells (0.014–0.077 nmol/min/10$^6$ cells), but the $K_{m}^{E_1S}$ was of high affinity (4–6 μM) and was similar in PP and PV cells. These values suggest that estrone sulfation, when compared with estrone sulfate desulfation, is a high-affinity but low-capacity pathway that would rapidly become saturated. By constraining the $K_{m}^{E_1S}$ as CL$^{E_1S}_{uptake} / V_{max}^{E_1S}$, we optimized the $V_{max}^{E_1S}$ value; the converse procedure of constraining the $V_{max}^{E_1S}$ as CL$^{E_1S}_{uptake} / K_{m}^{E_1S}$ resulted in a higher coefficient of variation for the estimate of $K_{m}^{E_1S}$. The $V_{max}^{E_1S}$ and $K_{m}^{E_1S}$ values for formation of other metabolites, M, were 5.9 to 9.4 nmol/min/10$^6$ cells and 18 to 19 μM, respectively, in PP and PV cells, showing that formation of other estrone metabolites greatly exceeds that of E$1_S$ and exhibits a greater PV abundance. These fitted results indicate that both the sulfation of E$1$ and the formation of M in PP hepatocytes are significantly higher than those in PP hepatocytes (Tables 4 and 5). The fitted tissue unbound fraction for E$1$ was low (0.025–0.03) and was likely due to the presence of the estrogen binding protein in hepatocytes (Rao, 1998).

The inclusion of the vesicular compartment in modeling seemed to be justified since cellular accumulation of E$1_S$ followed by only a gradual depletion of E$1_S$ was observed. Indeed, absence of the cellular storage compartment of E$1_S$ provided a slightly inferior fit, predicting a slightly faster decay of E$1_S$ and greater formation of E$1$ in the liver cell. The contents of E$1_S$ and E$1$ in the extracellular and total medium were, however, affected only slightly (data not shown), since the total accumulation of E$1_S$ in the vesicular space at 30 min amounted to only 2% of the dose. By contrast, the binding of E$1_S$ in debris and in tissue was found to be of paramount importance. Absence of binding resulted in very poor fits that predicted monoexponential decay rate constants for E$1_S$ in the extracellular medium, and the accumulation of E$1_S$ in the cell at early time points (Fig. 6C) was greatly attenuated in the absence of binding (data not shown). The accumulation pattern of E$1_S$ in cell is therefore attributed mostly to tissue binding and less to vesicular storage.

**Discussion**

Estrone sulfate plays a vital role in the cycling of estrogens. Being hydrophilic, E$1_S$ serves as a mobile estrogen and allows easy delivery to target tissues. E$1_S$, a common substrate of Oatp1, Oatp2, Oatp4, Ntcp, and OAT3, gains ready access into the liver tissue where it is metabolized, excretion of E$1_S$, the transmembrane characteristics, and tissue protein binding, recognizing that some of the proteins mediating the processes may be zonated in the acinus. Hence, we assessed the zonal, metabolic activities in tissue and integrated these with the transport and binding activities to examine the influence of metabolic heterogeneity on the futile cycling of estrogens in intact zonal hepatocytes. Notably, in contrast to parallel decay profiles in both extra-cellular medium for both parent and metabolite (Ebling and Jusko, 1986), we observed different decay half-lives for E$1_S$ in the extracellular medium, and the accumulation of E$1_S$ in the cell at early time points (Fig. 6C) was greatly attenuated in the absence of binding (data not shown). The accumulation pattern of E$1_S$ in cell is therefore attributed mostly to tissue binding and less to vesicular storage.

From in vitro values of the kinetic constants for E$1_S$ desulfation ($K_{in}^{E_1S=E_1}$ of 30 and 35 μM and $V_{max}^{E_1S=E_1}$ values of 2.2 and 1.9 nmol/min/10$^6$ cells, respectively, for PP and PV hepatocytes), there was no difference in metabolic activity for both the proximal and distal regions of the rat liver. The observation was in good agreement
with other findings on the homogeneous distribution of arylsulfatase C activity (Anundi et al., 1986). The $K_m$ values were similar to that of a previous study (32 $\mu$M; Iwamori et al., 1976). By contrast, estrone sulfation was of higher affinity ($K_m$ E1 of 4.4–5.9 $\mu$M) in both PP and PV regions, but the $V_{\text{max}}$ E1 was much lower in value and differed between PP and PV hepatocytes (0.014 – 0.077 nmol/min/10^6 cells, respectively; Table 4). The observation was consistent with the trends on sulfation of tracer estrone in both hepatocytes and lysates (Table 2) as well as with immunoblot analyses of rSULT1E1 (Figs. 3 and 4). The PP/PV ratio of rSULT1E1 protein was consistent with those reported by others (Tosh et al., 1996). But the low PV/PP ratio of rSULT2A1 was opposite to the observation on sulfation activities, suggesting that hydroxysteroid sulfotransferase contributes little to estrone sulfation. The even distribution of rSULT1A1 protein in the zonal cells also indicates that phenol sulfotransferase only plays a minor role in estrone sulfation. This evidence confirms that sulfation of estrone is predominantly catalyzed by estrogen sulfotransferase (rSULT1E1) in the presence of PAPS.

The $CL_{\text{int}}$ E1 was about 4 to 23 times higher than the $CL_{\text{ext}}$ E1 (Table 5), and E1 sulfation was the rate-limiting step in the futile cycling. In addition to E1 sulfation, E1 was metabolized to M with a much higher intrinsic clearance ($CL_{\text{int}}$ M, 328 and 495 $\mu$L/min/10^6 cells).
cells, respectively, for PP and PV cells; Table 5) that showed a PV preponderance. The competitive metabolism of E1 represents both glucuronidation by the UDP-glucuronosyltransferases that are localized pericentrally (Tosh and Burchill, 1996) and oxidation of E1 by CYP1A2 and -3A that are concentrated in the PV region (Oinonen et al., 1996). This “pooled” \( \text{CL}_{\text{m, E1 S}} \) was 38 to 106 times higher than the \( \text{CL}_{\text{m, E1}} \). Consequently, little E1 is resulfated back to form E1S. The higher activities for E1 sulfation and formation of M in the PV region translates to the higher accumulation of E1 in PP cells, as observed under low concentrations (cf. AUC values in Table 3).

Upon comparison of the metabolic intrinsic clearances of E1 sulfation and E1S desulfation to those for transport, the hepatic uptake clearances greatly exceed the metabolic intrinsic clearances (Table 5). The transport clearance of E1S is rapid, but that for E1 is even faster. The CL_{uptake} (Table 5) is substantial. Under physiological and first-order conditions where both E1 and E1S exist in low concentrations (nM), transport should remain very rapid and unsaturated. At high concentrations of E1S, however, transport may become saturated at concentrations comparable with or exceeding \( K_{m}^{E1S} \). The value of the fitted \( K_{m}^{E1S} \) is within the range of the \( K_{m} \) values (4.5–27 \( \mu M \)) reported for the various transporters and was similar to the value of \( K_{m}^{E1} \) (24 \( \mu M \)) obtained in vitro (Tan et al., 1999). Adoption of the in vitro \( K_{m}^{E1S} \) value (24 \( \mu M \)), however, provided poorer fits. We found that the parameters for the transport systems of E1S obtained from fitting were similar for both PP and PV hepatocytes, and the finding suggests the uniform distribution of transporters in rat liver. Uniform acinar distributions were found for Ntcp (Stieger et al., 1994), Oatp1 (Abuzahra et al., 2000), and Oatp2 (Tirona et al., 2000) in rat liver, and uptake of E1S was similar in zonal hepatocytes (Tan et al., 1999). Saturation in uptake had occurred within the concentration range studied in the hepatocyte system, and this was shown by the decreasing partition coefficients of E1S with increasing concentrations (Fig. 7B). Consistent with lack of zonation in uptake, values of the equilibrium partition coefficients of E1S were similar for both PP and PV hepatocytes.

Although previous evidence has suggested that transport of E1 across the membrane might involve carriers (Rao et al., 1977), our data were consistent with a linear, transmembrane flux for E1 (\( \text{CL}_{\text{E1}} \)). The bidirectional uptake clearance for E1 (1463–1484 \( \mu M/\text{min}/10^{6} \text{ cells} \)) was even faster than that for E1S, and no difference was found among PV and PP hepatocytes. The rapid transport clearance of E1 was congruent with parallel trends of E1 in cellular and extracellular

![Fig. 7. Partitioning of E1S between cell and medium in PP and PV hepatocyte systems.](image)

A, time-dependent profiles for the partition coefficients of E1S, cellular concentrations/extracellular concentration, in relation to the different initial concentrations of E1S [1 1 M \( (\bullet) \), 5 1 M \( (\bigcirc) \), 25 1 M \( (\blacktriangle) \), and 125 1 M \( (\text{triangle down}) \); mean \( \pm \) S.D., and the corresponding open symbols represent the PV data. B, a decreasing pattern of the partition coefficients of E1S existed at equilibrium for the various initial concentrations of E1S (1–125 \( \mu M \)).

### Table 3

<table>
<thead>
<tr>
<th>E1S ( \mu M )</th>
<th>Cellular</th>
<th>Extracellular</th>
<th>PV</th>
<th>AUC of E1S</th>
<th>Extracellular</th>
<th>PP</th>
<th>AUC of E1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC of E1S</td>
<td>( \mu M \cdot \text{min}/10^{6} \text{ cells} )</td>
<td>AUC of E1S</td>
<td>( \mu M \cdot \text{min}/10^{6} \text{ cells} )</td>
<td>AUC of E1S</td>
<td>( \mu M \cdot \text{min}/10^{6} \text{ cells} )</td>
<td>AUC of E1</td>
</tr>
<tr>
<td></td>
<td>( \text{AUC}_{E1S}^{\text{cell}}(0 \rightarrow 30 \text{ min}) )</td>
<td>( \text{AUC}_{E1S}^{\text{extracellular}}(0 \rightarrow 30 \text{ min}) )</td>
<td>( \text{AUC}_{E1S}^{\text{cell}}(0 \rightarrow \infty) )</td>
<td>( \text{AUC}_{E1S}^{\text{extracellular}}(0 \rightarrow \infty) )</td>
<td>( \text{AUC}_{E1}^{\text{cell}}(0 \rightarrow 30 \text{ min}) )</td>
<td>( \text{AUC}_{E1}^{\text{extracellular}}(0 \rightarrow 30 \text{ min}) )</td>
<td></td>
</tr>
<tr>
<td>PP</td>
<td>1</td>
<td>74 ± 31</td>
<td>2.6 ± 0.9</td>
<td>3.0 ± 1.1</td>
<td>0.34 ± 0.12</td>
<td>60 ± 27</td>
<td>0.71 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>560 ± 147</td>
<td>18 ± 3.3</td>
<td>20 ± 3.9</td>
<td>0.25 ± 0.05</td>
<td>300 ± 152</td>
<td>6.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3,668 ± 1,123</td>
<td>215 ± 27</td>
<td>236 ± 30</td>
<td>0.106 ± 0.014</td>
<td>2,052 ± 1,738</td>
<td>57 ± 14</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>18,704 ± 7,102</td>
<td>2,372 ± 325</td>
<td>2,634 ± 365</td>
<td>0.047 ± 0.007</td>
<td>5,363 ± 2,272</td>
<td>179 ± 70</td>
</tr>
<tr>
<td>PV</td>
<td>1</td>
<td>61 ± 14</td>
<td>2.5 ± 1.4</td>
<td>2.7 ± 1.6</td>
<td>0.37 ± 0.21</td>
<td>42 ± 29</td>
<td>0.35 ± 0.10*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>432 ± 161</td>
<td>17 ± 8.2</td>
<td>19 ± 9.1</td>
<td>0.26 ± 0.12</td>
<td>222 ± 113</td>
<td>3.7 ± 0.94*</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3,366 ± 1,340</td>
<td>212 ± 75</td>
<td>227 ± 81</td>
<td>0.11 ± 0.04</td>
<td>1,755 ± 1,208</td>
<td>42 ± 15</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>18,908 ± 4,489</td>
<td>2,363 ± 322</td>
<td>2,586 ± 364</td>
<td>0.048 ± 0.007</td>
<td>5,166 ± 2,400</td>
<td>174 ± 45</td>
</tr>
</tbody>
</table>

* Statistically different from periporal data (ANOVA, \( P < 0.05 \)).

\( ^{b} \) \( \text{CL}_{\text{app}} = [\text{dose} / \text{AUC}_{E1S}^{\text{cell}}(0 \rightarrow \infty)] \).

\( ^{b} \) AUC of 0 to 30 min, estimated by trapezoidal method.

\( ^{b} \) AUC of 0 to 30 min, estimated using trapezoidal method, and this was added to \( C_{\text{cell}} / k \) (concentration at 30 min divided by the first order decay constant, \( k \)), assuming log-linear decline.
of E1 S and E1, whereas cellular binding of E1 S and E1 entraps the species binding exists (Fig. 5B). Extracellular binding would decrease the uptake within the cell and impedes cellular elimination. Tissue binding of E1 S and E1 has led to the conclusion that an even tighter tissue binding, and the presence of vesicular accumulation of E1 S. When only linear transport was introduced, linear 

### TABLE 4

**Assigned and fitted parameters for the cellular kinetic model of E1 S and E1 in zonal hepatocytes**

<table>
<thead>
<tr>
<th>Parameters Description</th>
<th>Periportal</th>
<th>Perivenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>$v_{max}$</td>
<td>Maximum uptake velocity for E1 S (nmol/min/10^6 cells)</td>
<td>2.9</td>
</tr>
<tr>
<td>$K_{m}$</td>
<td>Michaelis-Menten constant for E1 S uptake (μM)</td>
<td>11.8 ± 0.9</td>
</tr>
<tr>
<td>$p_{diff}$</td>
<td>Bidirectional uptake clearance for E1 S (μl/min/10^6 cells)</td>
<td>4.0</td>
</tr>
<tr>
<td>$p_{diff}$</td>
<td>Bidirectional uptake clearance for E1 (μl/min/10^6 cells)</td>
<td>1484 ± 242</td>
</tr>
<tr>
<td></td>
<td>Maximum desulfation rate for E1 S (nmol/min/10^6 cells)</td>
<td>2.2</td>
</tr>
<tr>
<td>$K_{m}$</td>
<td>Michaelis-Menten constant for desulfation of E1 S (μM)</td>
<td>30.4</td>
</tr>
<tr>
<td>$v_{max}$</td>
<td>Maximum sulfation rate for E1 S (nmol/min/10^6 cells)</td>
<td>0.014 ± 0.015</td>
</tr>
<tr>
<td>$K_{m}$</td>
<td>Michaelis-Menten constant for sulfation of E1 (μM)</td>
<td>4.4</td>
</tr>
<tr>
<td>$v_{max}$</td>
<td>Maximum metabolism rate for E1 S (nmol/min/10^6 cells)</td>
<td>5.9 ± 2.0</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Number of binding sites for E1 S</td>
<td>1.5</td>
</tr>
<tr>
<td>$K_{on}$</td>
<td>Binding association rate constant for E1 S (l/min/μM/10^6 cells)</td>
<td>0.0014 ± 0.0001</td>
</tr>
<tr>
<td>$V_{off}$</td>
<td>Binding dissociation rate constant for E1 S (l/min/10^6 cells)</td>
<td>0.033</td>
</tr>
<tr>
<td>$P_{diff}$</td>
<td>Unbound fraction of intracellular E1</td>
<td>0.030 ± 0.005</td>
</tr>
<tr>
<td>$P_{diff}$</td>
<td>Unbound fraction of extracellular E1</td>
<td>0.84</td>
</tr>
<tr>
<td>$V_{int}$</td>
<td>Cellular volume (ml/10^6 cells)</td>
<td>0.0036</td>
</tr>
<tr>
<td>$V_{int}$</td>
<td>Extracellular volume (ml/10^6 cells)</td>
<td>0.9964</td>
</tr>
</tbody>
</table>

* Statistically different from parameter for periportal hepatocytes (ANOVA, P < 0.05).
* Obtained from Tan et al. (1999) and scaled up as described under Experimental Procedures.
* Scaled up from the desulfation rate constants for E1 S, obtained from S9 of zonal hepatocytes.
* Constrained as $V_{max} = \frac{V_{max} \cdot E1_{int}}{K_{m}}$.
* Glucuronidation, hydroxylation, and oxidation of E1 (other than sulfation of E1).
* Division of the effective binding concentration of E1 S by the extracellular protein concentration.
* Constrained as $K_{on} \cdot K_{diff}$ multiplied by $K_{diff}$.
* Unbound fraction of E1 in the extracellular medium.

* Obtained from Garcia-Ruiz et al. (1994).

### TABLE 5

**Comparison of the clearances for E1 S and E1 in zonal hepatocytes**

<table>
<thead>
<tr>
<th>Parameters Description</th>
<th>Periportal</th>
<th>Perivenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{L,E1 S}$</td>
<td>Uptake clearance for E1 S</td>
<td>246</td>
</tr>
<tr>
<td>$C_{L,E1}$</td>
<td>Uptake clearance for E1</td>
<td>1484</td>
</tr>
<tr>
<td>$C_{L,E1 S - E1}$</td>
<td>Intrinsic clearance for E1 S desulfation</td>
<td>72</td>
</tr>
<tr>
<td>$C_{L,E1 S}$</td>
<td>Intrinsic clearance for sulfation of E1 S</td>
<td>3.2</td>
</tr>
<tr>
<td>$C_{L,E1}$</td>
<td>Intrinsic clearance for glucuronidation, hydroxylation, and oxidation of E1 (other than sulfation of E1)</td>
<td>328</td>
</tr>
<tr>
<td>$C_{L,E1 S}$</td>
<td>Intrinsic clearance for accumulation of E1 S into vesicle</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* Values become ml/min/g of liver when multiplied by the factor (125/1000).
* Obtained as $C_{L,E1 S} = \frac{v_{max} \cdot K_{m}}{V_{max} \cdot E1_{int} + K_{diff}}$.
* $\frac{v_{max} \cdot K_{m}}{V_{max} \cdot E1_{int} + K_{diff}}$.
* $\frac{v_{max} \cdot K_{m}}{V_{max} \cdot E1_{int} + K_{diff}}$.
* $\frac{v_{max} \cdot K_{m}}{V_{max} \cdot E1_{int} + K_{diff}}$.

* Obtained from Tan et al. (1999) and scaled up as described under Experimental Procedures.
* Constrained as $V_{max} = \frac{V_{max} \cdot E1_{int}}{K_{m}}$.
* Constrained as $K_{on} \cdot K_{diff}$ multiplied by $K_{diff}$.
* Unbound fraction of E1 in the extracellular medium.

This study is the first account on binding of substrates to cell debris that resulted during hepatocyte preparation. The presence of extracellular binding of E1 S and E1 has led to the conclusion that an even tighter tissue binding exists (Fig. 5B). Extracellular binding would decrease the uptake of E1 S and E1, whereas cellular binding of E1 S and E1 entraps the species within the cell and impedes cellular elimination. Tissue binding of E1 S and E1 therefore exerts a significant influence on the cellular kinetics of futile cycling of estrogens. Another issue that needs to be addressed with respect to tissue binding and metabolism is nonlinear tissue binding of E1 S and $K_{inf}^{E1 S} < K_{inf}^{E1 S-E1}$ (24 vs. 30–34 μM). The comparison of $K_{m}$ values suggests that, with increasing cellular concentrations of E1 S, saturation of tissue binding precedes the saturation of the metabolic enzymes for desulfation. A similar scenario—with $K_{m}$ for vascular binding of a flow-limited substrate < the $K_{m}$—had resulted in nonlinearity in drug clearance (Chiba and Pang, 1993; Xu et al., 1993). The same consequence will result here with nonlinearity in tissue binding.

To understand the interplay among the nonlinearity in transport, tissue binding, and the presence of vesicular accumulation of E1 S on the different $t_{1/2}$ values of E1 S and of E1, simulations were further performed with the fitted parameters, with the substitution a single, nonsaturable uptake clearance of E1 S ($C_{L,Uptake}$ of 246 μl/min/10^6 cells), then a 10× higher dissociation binding constant ($K_{diff}^{E1 S}$ was increased to 230 μM), and ultimately an absence of vesicular accumulation of E1 S. When only linear transport was introduced, linear decay of extracellular E1 S was observed. But the difference in $t_{1/2}$ values of E1 S and E1 persisted (data not shown). The similarity in decay $t_{1/2}$ values of E1 S and of E1 for any given dose could only be attained when $C_{L,Uptake}$ was high and linear (246 μl/min/10^6 cells), when $K_{inf}^{E1 S}$ greatly exceeded $K_{inf}^{E1 S-E1}$, and when there was a lack of vesicular accumulation of E1 S. The extracellular and cellular E1 S contents would now decay in unison with those of E1, and similar
half-lives were attained for both drug and metabolite species, as expected of the futile cycling phenomenon (Fig. 8). The pattern conforms to other reversible metabolic systems that describe the futile cycling between methylprednisolone and methylprednisone for which similar in vivo elimination half-lives were observed for both drug and metabolite (Ebling and Jusko, 1986). It may be thus concluded that the nonlinearity in uptake and tissue binding, and the presence of vesicular accumulation of \( E_1S \), had resulted in different decay half-lives for \( E_1S \) and \( E_1 \) in the hepatocyte system.

In conclusion, both \( E_1 \) and \( E_1S \) are rapidly taken up evenly into rat zonal hepatocytes. The sulfation of \( E_1 \) by estrogen sulfotransferase and the metabolism of estrone to other metabolites were more abundant in PV than in PP hepatocytes, although the desulfation of \( E_1S \) was evenly distributed. The rate-limiting factor for the futile cycling of \( E_1S \) and \( E_1 \) was sulfation, since transport was rapid and the intrinsic clearance of \( E_1S \) desulfation was higher than that of \( E_1 \) sulfation. The higher levels of \( E_1 \) and \( E_1S \) in PP hepatocytes were due to the higher PV metabolic activity towards \( E_1 \) sulfation and the formation of other metabolites. Different decay half-lives for \( E_1 \) and \( E_1S \) were observed, which were attributable to nonlinear uptake, tissue binding, and vesicular uptake of \( E_1S \) in the cell.

**Fig. 8.** Simulated profiles of \( E_1S \) and \( E_1 \) in the hepatocyte incubation system.

Profiles were based on the PP parameters and substitution of a single, linear transport clearance of \( E_1S \) (\( CL_{uptake}^{E_1S} \) was set constant at 246 \( \mu l/min/10^6 \) cells), then a \( 10^3 \) higher binding dissociation constant (\( K_{D}^{E_1S} \) was increased to 230 \( \mu M \)), and absence of vesicular accumulation of \( E_1S \). The concentrations of \( E_1S \) and \( E_1 \) associated with the four numbered initial concentrations of \( E_1S \) (1, 5, 25, and 125 \( \mu M \)) are shown. A, total concentrations of \( E_1S \); B, extracellular concentrations of \( E_1S \); C, cellular concentrations of \( E_1S \); D, total concentrations of \( E_1 \); E, cellular concentrations of \( E_1 \); and F, extracellular concentrations of \( E_1 \) were presented. The simulated lines for 1 (--), 5 (---), 25 (-----), and 125 \( \mu M \) (-----) of \( E_1S \) were based on eqs. A1 to A11 (Appendix).
Appendix

A cellular kinetic model was presented (Fig. 1). [E₁S], [E₁], [M], and [P] denote the concentrations of E₁S, E₁, metabolites of E₁ other than E₁S, and protein in various compartments; subscripts esc, c, and ves represent the extracellular medium, the cellular space, and vesicular compartment, respectively. Parameters were described in Table 3 and under Experimental Procedures.

The equations describing extracellular space (ec) for E₁ and E₁S are as follows:

\[
\frac{d[E₁S\text{ unbound}_{ec}]}{dt} = \left( p^{E₁S\text{ unbound}}_{\text{diff}} [E₁S\text{ unbound}]_{ec} + p^{E₁S\text{ unbound}}_{\text{conv}} [E₁S\text{ unbound}]_{ec} \right) V_{ec} - \left( k^{E₁S\text{ unbound}}_{\text{off}} + k^{E₁S\text{ unbound}}_{\text{on}} \right) [E₁S\text{ unbound}]_{ec} V_{ec} \]  

(A1)

The total concentration of extracellular E₁S is the sum of unbound and bound E₁S and is given by

\[
\frac{d[E₁_{\text{total}}]}{dt} = \left( p^{E₁}_{\text{diff}} [E₁]_{ec} [E₁S_{\text{bound}}]_{ec} + p^{E₁}_{\text{diff}} [E₁]_{ec} [E₁S_{\text{unbound}}]_{ec} \right) V_{ec} - \left( k^{E₁S\text{ unbound}}_{\text{off}} + k^{E₁S\text{ unbound}}_{\text{on}} \right) [E₁S_{\text{unbound}}]_{ec} V_{ec} \]  

(A3)

The equations describing cellular space (c) for E₁ and E₁S, are

\[
\frac{d[E₁S_{\text{unbound}}]}{dt} = \left( k^{E₁S\text{ unbound}}_{\text{on}} [P_{\text{total}}]_{ec} [E₁S_{\text{bound}}]_{ec} - k^{E₁S\text{ unbound}}_{\text{off}} [E₁S_{\text{bound}}]_{ec} \right) V_{c} \]  

(A2)

\[
\frac{d[E₁S_{\text{bound}}]}{dt} = \left( p^{E₁S\text{ bound}}_{\text{diff}} [E₁S_{\text{bound}}]_{ec} + p^{E₁S\text{ bound}}_{\text{conv}} [E₁S_{\text{bound}}]_{ec} \right) V_{c} - \left( k^{E₁S\text{ unbound}}_{\text{off}} + k^{E₁S\text{ unbound}}_{\text{on}} \right) [E₁S_{\text{unbound}}]_{ec} V_{c} \]  

(A5)

The total amount of intracellular E₁S is the sum of unbound, bound, and vesicular contents of E₁S, and the total concentration of intracellular E₁S is obtained by dividing the total amount of intracellular E₁S by the cellular volume.

\[
\frac{d[E₁_{\text{total}}]}{dt} = \left( p^{E₁}_{\text{diff}} [E₁]_{ec} [E₁S_{\text{bound}}]_{ec} + p^{E₁}_{\text{diff}} [E₁]_{ec} [E₁S_{\text{unbound}}]_{ec} \right) V_{c} - \left( k^{E₁S\text{ unbound}}_{\text{off}} + k^{E₁S\text{ unbound}}_{\text{on}} \right) [E₁S_{\text{unbound}}]_{ec} V_{c} \]  

(A7)

The metabolic intrinsic clearance for desulfation is

\[
CL_{\text{int}} = \frac{[E₁S]_{\text{total}} - [E₁S]_{\text{bound}}}{[P_{\text{total}}]_{ec}} \]  

(A9)

The metabolic intrinsic clearance for desulfation is

\[
k^{E₁S}_{\text{on}} \frac{[P_{\text{total}}]_{ec}}{K^{E₁S}_{\text{on}}} \]  

(A10)

The metabolic intrinsic clearance for desulfation is

\[
k^{E₁S}_{\text{on}} \frac{[P_{\text{total}}]_{ec}}{K^{E₁S}_{\text{on}}} \]  

(A11)

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